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Antitumor Spectra of Anthracyclines Against Gastric Cancer Tissues Obtained From Surgical Specimens With Reference to P-Glycoprotein Expression

TOMOKO FURUKAWA,[1]* TETSURO KUBOTA, MD,[2] HIROYUKI MURATA,[1] HIROKAZU TANINO, MD,[3] SATOKO YUASA,[1] KAZUE MORITA,[1] JUNJI UENO,[1] KATSUMI KOZAKAI,[1] AND TAKASHI YANO, PhD[1]

[1]*Immunochemical Laboratory, Eiken Chemical Co., Ltd. Tokyo, Japan*
[2]*Department of Surgery, School of Medicine, Keio University, Tokyo, Japan*
[3]*Department of Thoracic and Cardiovascular Surgery, Wakayama Medical College, Wakayama, Japan*

Background and Objectives: Although the mechanism of P-glycoprotein (Pgp)-related resistance of doxorubicin is known, it has not been clarified for other anthracycline derivatives. We have examined the chemosensitivity of gastric cancer tissues to three anthracyclines in relation to Pgp expression.

Methods: Sixty-six surgical specimens obtained from patients with gastric cancer were subjected to histoculture drug response assay using doxorubicin (DXR), epirubicin (EPI), and 4′-*O*-tetrahydropyranyldoxorubicin (pirarubicin; THP). The cutoff concentrations used were 15 μg/ml for DXR and EPI and 17 μg/ml for THP.

Results: A 50% or more inhibition index (I.I.) was regarded as sensitive, at which the correlation rates were 95.8% (23/24) and 74.2% (49/66) for DXR-EPI and DXR-THP, respectively. Twenty-six specimens were immunohistochemically stained with monoclonal antibody to Pgp, with a positive rate of 53.8% (14/26). In Pgp-positive specimens, all cases were resistant to DXR and 28.6% (4/14) of cases were sensitive to THP, while the antitumor activity of EPI was essentially identical to that of DXR.

Conclusions: The expression of Pgp might affect resistance to DXR and EPI, although THP may partially impair this resistance, suggesting the clinical usefulness of THP in treatment of DXR-refractory gastric carcinoma. *J. Surg. Oncol. 1998;69:173–177.* © 1998 Wiley-Liss, Inc.

KEY WORDS: **P-glycoprotein; gastric cancer; doxorubicin; pirarubicin; histoculture drug response assay; chemosensitivity**

INTRODUCTION

Anthracyclines, including doxorubicin (DXR), have been used extensively to treat solid tumors for many years. There have been many studies on the resistance of anthracyclines pumped out from the cancer cells by P-glycoprotein (Pgp) expressed on the cell membrane. Pgp is encoded by the multidrug resistance gene; mdr-1 [1–3]. Epirubicin (EPI) and 4′-*O*-tetrahydropyranyl doxorubicin (pirarubicin; THP) were developed as the second-generation anthracyclines with higher or similar cytotoxicity and fewer side effects in terms of cardiotoxicity and alopecia compared with DXR.

In vitro chemosensitivity tests have been conducted using fresh surgical specimens from solid tumors for

*Correspondence to: Tomoko Furukawa, Immunochemical Laboratory, Eiken Chemical Co., Ltd., 5-26-20, Oji, Kita-ku, Tokyo 114-0002, Japan. Fax No.: (81) 3-3913-6201.
E-mail: tomokof@pb3.so-net.ne.jp

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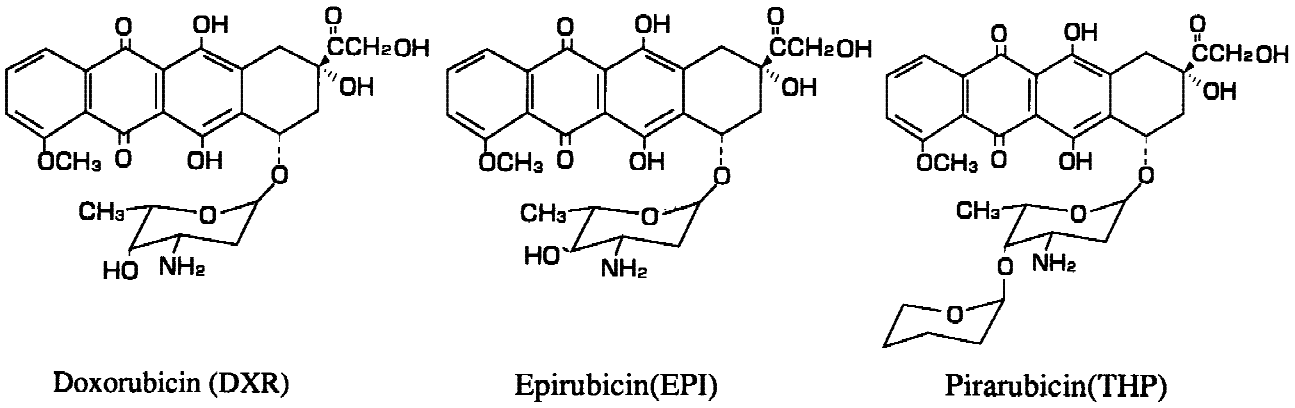

Fig. 1. Chemical structures of anthracycline derivatives.

some 30 years. Mossman [4] reported 3-(4,5-dimethylthiazolyl)-2,5-diphenyl 2H-tetrazolium bromide (MTT) assay as a simple and convenient colorimetric assay, and histoculture drug response assay (HDRA) with the endpoint of ^{3}H-thymidine incorporation has been reported by Hoffman et al. [5,6], techniques that allow the tumor specimens to maintain cell-to-cell contact and three-dimensional tissue architecture. Furthermore this assay was improved by Furukawa et al. [7], who adopted the MTT assay as its endpoint and reported high predictability for clinical outcome of cancer chemotherapy [8,9].

In the present study, we have evaluated the sensitivity of the surgical specimens obtained from patients with gastric cancer to anthracyclines, including DXR, EPI, and THP, comparing the sensitivity of these drugs with Pgp expression.

MATERIALS AND METHODS

Drugs

Doxorubicin (DXR) and epirubicin (EPI) were purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan; pirarubicin (THP) was purchased from Nippon Kayaku Co., Ltd., Tokyo, Japan. The chemical structures of these compounds are shown in Figure 1.

Histoculture Drug Response Assay

The specimens were obtained from 66 patients with gastric cancer (all tubular adenocarcinomas), including 62 primary, 1 lymph node metastasis, 2 hepatic metastasis, and 1 peritoneal dissemination cases. The test samples were removed from the surgical specimens under aseptic conditions and transported in Hanks' balanced salt solution (HBSS; GIBCO, Gaithersburg, MD) to our laboratory.

Histoculture drug response assay (HDRA) was performed according to a method previously reported [9]. The anticancer drugs were dissolved in RPMI 1640 medium (Sigma, St. Louis, MO) containing 20% fetal calf serum (Uniglobe Research, Reseda, CA), penicillin-streptomycin-amphotericin B (GIBCO), 100 units/ml, 100 μg/ml, and 0.25 μg/ml, respectively. One milliliter per well of the solution was poured into a 24-well plate. The cutoff concentration used for DXR, 15 μg/ml, was chosen based on our previous report [9], with equivalent molar concentrations used for the cutoff concentrations of EPI (15 μg/ml) and THP (17 μg/ml), respectively. The collagen gel (Gel Foam®; Pharmacia & Upjohn, UK) was cut into 1-cm pieces and placed into the wells of the plates.

The surgical specimens were cut into 10–15 mg pieces, weighed by chemical balance (R200D: Sartorius, Germany), and placed onto the collagen gels. Six and four replicates were run for control and treatment groups, respectively. After incubation for 7 days, 100 μl of 0.06% collagenase (type I; Sigma) solution in HBSS, and 100 μ1 of 0.2% MTT (Sigma) phosphate buffered saline (PBS) solution, containing 50 mM sodium succinate (Wako Pure Chemical Industries, Tokyo, Japan), were added to each well. After the plates were incubated for a further 16 hr, the medium was removed and 0.5 ml per well of dimethyl sulfoxide (DMSO) was added to extract MTT-formazan. After 2 hr, extracted solution of each well (100 μl) was moved to 96-well plate, and the absorbance measured with a microplate reader (MTP-120: Corona Electric, Ibaraki, Japan) at 540 nm (reference 630 nm). Cases of contamination and absorbance of less than 15 per 1 g control tumor were regarded as unevaluable.

Inhibition index was calculated as follows: Inhibition index (%) = $(1 - A/B) \times 100$, where A is mean absorbance of the treated wells per 1 g tumor, and B is mean absorbance of the control wells per 1 g tumor. A drug with a 50% or higher inhibition index was regarded as in vitro sensitive.

Immunohistochemical Staining

JSB-1, a murine monoclonal antihuman P-glycoprotein (Pgp: Nichirei, Tokyo, Japan) was used for the study. Immunohistochemical staining was performed

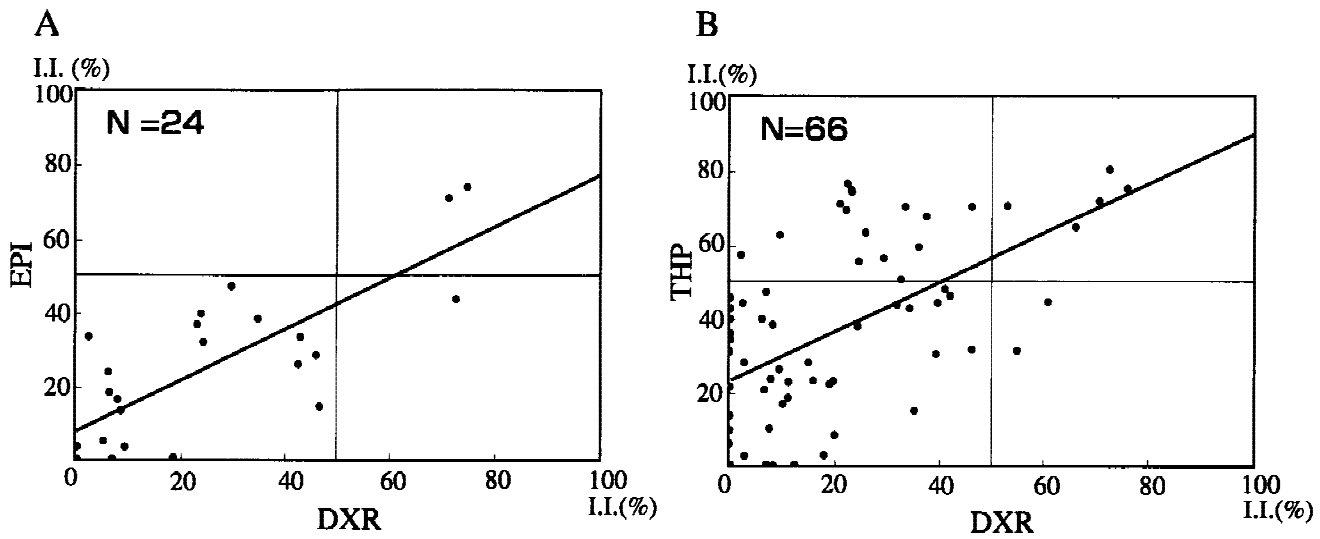

Fig. 2. Similarity of antitumor spectra of anthracyclines. **A:** Doxorubicin (DXR)-epirubicin (EPI). **B:** Doxorubicin (DXR)-pirarubicin (THP). The inhibition indexes were compared between DXR-EPI (A) and DXR-THP (B). The coefficient of correlation between DXR-EPI (0.782) was higher than that between DXR-THP (0.582). I.I. denotes inhibition index; N, number of cases; EPI, epirubicin; DXR, doxorubicin; THP, pirarubicin.

according to the standard streptavidin-biotin peroxidase complex method using a Histofine® SAB-PO[M] kit (Nichirei). In brief, the deparaffinized paraffin sections were placed in methanol containing 1% hydrogen peroxide for 15 min to block endogenous peroxidase activity. After washing with PBS, sections were incubated with antibody of anti-Pgp, diluted in X60 for 16 hr at 4°C in a moist chamber, then incubated with biotinylated rabbit antimouse immunoglobulin G and peroxidase-conjugated streptavidin, each for 30 min at room temperature. After the final washing with PBS, sections were immersed in a PBS solution containing 0.02% 3,3′-diaminobenzidine and 0.1% hydrogen peroxide. The reacted sections were stained with 0.1% hematoxylin and mounted with balsam. The PC14/TXT cell line was used as a positive control of Pgp-staining. The tested specimens were regarded as Pgp-positive when 30% or more of tumor cells were stained with anti-Pgp.

Statistical Analysis

The statistical analysis was performed according to the Student *t*-test or chi-square test. *P* was regarded as significant at <0.05.

RESULTS

Correlation Between Sensitivity to DXR and EPI or THP

All cases were evaluable in histoculture drug response assay. Figure 2 shows the correlation between the inhibition index of DXR and those of THP and EPI, respectively. The coefficient of correlation between DXR-EPI (0.782) was higher than that between DXR-THP (0.582). Fifteen cases resistant to DXR were evaluated as THP-sensitive, while only two cases were DXR-sensitive and THP-resistant. As a result, the efficacy rate of THP (30.3%; 20/66) was significantly higher than that of DXR (10.6%; 7/66) at $P < 0.05$ (one-tailed test), although there was no significant difference between those of DXR and EPI (8.3%; 2/24) by chi-square test.

Comparison of P-Glycoprotein Expression and DXR and THP Sensitivity

Twenty-six of 66 specimens were large enough for Pgp evaluation. Fourteen of 26 specimens stained positively with anti-Pgp monoclonal antibody, with a positive rate of 53.8% (14/26). A sample of the immunohistochemically stained specimen regarded as positive, is shown in Figure 3. In the Pgp-positive group, the efficacy rates were 0% (0/14) for DXR and 28.6% (4/14) for THP, while 25% (3/12) of specimens were sensitive to DXR and THP in Pgp-negative cases (Fig. 4). Resistance to DXR was significantly correlated with the expression of Pgp, at $P < 0.05$ (one-tailed test) by chi-square test, although this seemed not to relate closely with THP. Furthermore, the efficacy rate of THP was significantly higher than that for DXR in the Pgp-positive group at $P < 0.05$ (one-tailed test) by chi-square test, but not in the Pgp-negative group (Fig. 4).

DISCUSSION

In the present study, the in vitro sensitivity of gastric cancer specimens was assessed by histoculture drug response assay (HDRA) to anthracyclines. We have previously reported a 90% in vitro–in vivo correlation of drug response using human tumor xenografts and HDRA [7].

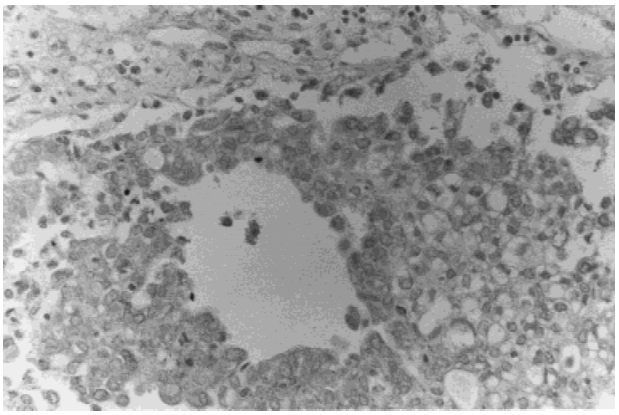

Fig. 3. Immunohistochemically stained specimen regarded as positive for P-glycoprotein (10 × 20). The staining method is described in the text. The positive reactions can be seen in the plasma membranes.

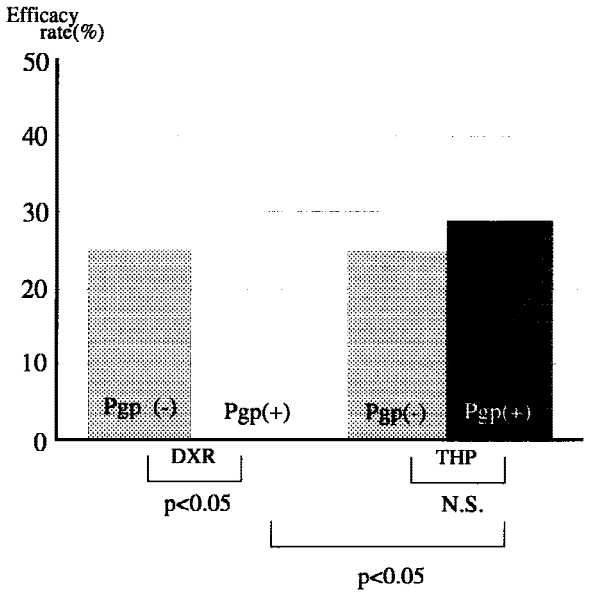

Fig. 4. Comparison of P-glycoprotein expression and sensitivity of doxorubicin or pirarubicin. In the Pgp-positive group, the efficacy rates were 0% for DXR and 28.6% for THP, while 25% of specimens were sensitive to DXR and THP in Pgp-negative cases. N.S. denotes not significant.

In this study, the efficacy of DXR significantly correlated with those of THP and EPI, although the efficacy rate for THP was higher than that for DXR or EPI. This suggested that these anthracyclines had a similar antitumor spectrum on fresh surgical specimens of gastric cancer tissues, while the efficacy rate for THP was higher than that for DXR or EPI when they were tested at the same molar concentration. The cutoff concentration of the drugs in the chemosensitivity test is estimated from pharmacokinetic data of tested drugs. Nakajima et al. [10] actually compared the pharmacokinetics of DXR and THP in patients with various cancers. When the same dose was administered on the different occasions, the serum concentration of DXR was higher than that of THP, while the distribution volumes of THP were larger than that of DXR. Saika et al. [11] observed that the tissue concentration of THP was increased compared with DXR in the bladder tumors when the drugs were instilled into the bladder, and the uptake of THP into cultured bladder cell lines, T-24, T-24/ADM was higher than that of DXR [12]. These results are consistent with our present finding that THP has higher antitumor activity than DXR at the same molar concentration.

Although the different resistant mechanisms of anthracyclines have been studied using cultured cell lines, fresh surgical specimens have been used for the in vitro chemosensitivity test in only one study by Salmon et al. [13], who investigated the immunohistochemically stained Pgp expression and sensitivity to DXR using fesh tumor tissues from patients with multiple myeloma, malignant lymphoma, or metastatic breast cancer. In their report, although only 3 of 14 Pgp-negative tumors exhibited in vitro resistance to DXR, all 12 fresh tumors that stained positive for Pgp were resistant to DXR at a significant level of $P < 0.001$. This result was comparable with our present finding that the efficacy rate of DXR on Pgp-negative gastric cancer specimens was 25%, while no cases were regarded as DXR-sensitive among the Pgp-positive group. On the other hand, the sensitivity to THP was not influenced by Pgp expression, suggesting that the mechanism of drug resistance of THP is not closely associated with the expression of Pgp. There were no significant differences between the efficacy rates for DXR and THP in Pgp-negative cases. Thus, it was suggested that the resistance involving Pgp is less important for THP than for DXR or EPI.

Shibata et al. [14] in a study using murine P388 leukemia cells and DXR-resistant P388/DXR cells reported that P388/DXR were 100-fold more resistant than the wild-type P388 cells, while its resistance to THP was limited to 50 times that of P388 cells. This result suggested that THP was more effective on DXR-resistant cells. These results are consistent with our present finding that THP was effective on DXR-resistant gastric cancer tissues obtained from fresh surgical specimens. The chemical structure of THP differs from that of DXR in a hydroxy group at the 4′ position, where a hydroxy moiety is substituted by a pyranyl ring, while EPI has only a small modification: a diametrical inversion at the 4′ position of the hydroxy group. Since it was suggested that the hydroxy group at the 4′ position is involved in the recognition of the drug to be bound to Pgp, the substitution of the *O*-pyranyl ring was thought to be relevant to breaking Pgp-related drug resistance (Fig. 1). Nagasawa et al. [15] reported efflux of THP in HL60 and THP-resistant HL60/THP cells without an expression of mdr-1 mRNA. In THP-resistant HL60/THP, a specific inhibitor of multidrug resistance–associated protein inhibited the

THP from the cells, while Pgp inhibitors did not inhibit this THP efflux. These results suggested that THP has a different mechanism of resistance from Pgp, as shown in the present study.

In conclusion, the present study demonstrated that the antitumor spectrum of EPI is essentially identical to that of DXR in gastric cancer tissues, and the mechanism of resistance of these drugs may be affected by Pgp expression. It further suggests that THP partly negates the resistance to efflux by Pgp.

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